THE UTILIZATION OF SOME CARBOHYDRATES BY IN VITRO CULTURED CHICK BLASTODERMS IN WOUND HEALING [1, 2]

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Studies by Spratt (1949a, 1949b, 1950a, 1950b) have shown us that there are rather specific requirements for differentiation and form building in the early chick embryo. By the use of chemically defined media he has been able to show the ability of the blastoderms to utilize different carbohydrate energy sources in development. One of the general conclusions drawn from this work is that there are specific nutritional requirements for many of the normal processes of development.

Former work, to be published elsewhere, devoted to the mechanical aspect of wound healing in the chick, has shown that this process is essentially of a morphological nature. When blood carbon is applied around holes of variable sizes produced in the extra-embryonic tissues of 24-hour blastoderms, it is observed that those particles placed immediately at the periphery of the wounds converge during closure of the holes, and come to lie within a very small area upon completion of healing. The distance that the carbon-marked cells move toward the center of the wound is inversely proportional to their initial distance from the margin, until a point is reached, beyond which the cells move outward instead of inward. The implication from such behavior is that the closure process is effected by a mass movement of cells, rather than by an unusually high cell proliferation at the borders of the wounds. This conclusion has been verified by observing in prepared slides that there is no difference in mitotic counts at any region around the sites of the injuries.

The present study represents an extension of the general problem of wound healing in the early chick blastoderm to include certain nutritional considerations. It is the purpose here to determine the ability of these organisms to utilize various media of known chemical composition for (1) the closure of wounds (for gross tissue movements in the extra-embryonic region) and (2) the closure of wounds in comparison to development in the embryo proper. It might be assumed that there would be greater requirements by the rapidly developing embryonic region.

Materials and Methods

All of the blastoderms used in the present study were of twenty-two hours incubation (head process to head fold stages). They were removed from the yolk, freed from the vitelline membrane and trimmed in a manner described in detail by Spratt (1947). By the use of sharp steel needles small, approximately

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square holes measuring 300 μ to 400 μ on a side were cut through all tissues in the pellucid area (Fig. 2). Following injury the embryos were transferred to the medium (see below) contained in watch glasses placed on moist cotton rings within petri dishes. Camera lucida diagrams were made at the time of injury and after intervals during subsequent incubation as indicated in the experiments, to note the degree of wound closure and the development of the embryo. Two hundred and thirty-four blastoderms were used in the study.

The preparation of the various sugar media has been described by Spratt (1949a). By volume the constituents were: chick Ringer solution (77.5%), penicillin-streptomycin solution (10%), phenol red (5%), phosphate buffer (5%), bicarbonate buffer (2.5%), sugar (quantity in mg% varying in the experiments); 425 mg. agar per 100 ml. total volume of medium were used.

The solutions utilized were prepared as follows:

Chick Ringer solution. 0.9 per cent NaCl, 0.042 per cent KCl, 0.024 per cent $CaCl_2$ in distilled water.

Penicillin-streptomycin solution. To 100,000 units penicillin-G-potassium and 20 mg. dihydro streptomycin sulfate (Squibb) add 20 ml. sterile Ringer saline. Refrigerated (frozen) this is claimed to be bacteriologically effective for one week.

Phenol red. 0.001 per cent solution in Ringer.

Phosphate buffer. Add 100 ml. distilled water to 0.290 gm. $Na_2HPO_4 - 12H_2O$ + 0.052 gm. $KHPO_4$. Autoclave to sterilize.

Bicarbonate buffer. Saturate a solution of 1.100 gm. $NaHCO_3$ in 100 ml. distilled water with CO_2. This is accomplished by blowing expired air into the solution through a tube for an extended period of time (ca. one hr.). Sterilize by filtration.

Sugar. Stock solution consists of 400 mg. monosaccharide per 100 ml. Ringer solution (400 mg% = 0.022 *M*). This was diluted to the desired concentration in the total volume of other medium ingredients. As an example, in preparing 40 ml. of 100 mg% glucose medium the following would be incorporated: Ringer (21 ml.), glucose stock solution (10 ml.), penicillin-streptomycin (4 ml.), phenol red (2 ml.), phosphate buffer (2 ml.), bicarbonate buffer (1 ml.), agar (170 mg.).

In making the media the agar, Ringer solution, phenol red and sugar preparation were combined and autoclaved. On cooling to approximately 45° C. the penicillin-streptomycin and sterile phosphate and bicarbonate buffers were added. After mixing by swirling this was poured into the watch glasses, where gelation occurred.

Aseptic technique was used throughout the experiments. The antibiotics were used as a precautionary measure. It was essential to guard against bacterial contamination, a factor not prominent when using an albumen medium. All of the equipment was dry-sterilized at 350° C. for 1.5 hours.

For the purpose of determining the nutritional requirements for wound healing in the blastoderms the following sugar concentrations were used: glucose, 100 mg%, 50 mg%, 10 mg% and 5 mg%; fructose, 100 mg% and 50 mg%; galactose, 50 mg%. Embryos explanted on a non-nutrient medium (all constituents except the carbohydrate) served as controls.

Results

The results of the experiments may be seen in Table I.

It will be observed that there was no healing except in one case in the controls on a medium lacking a carbohydrate substrate. This would imply that there was insufficient endogenous utilizable material for the necessary cell movements in wound healing. It was apparent that the embryos could succeed very well in

Table I

Nutritional requirements for wound healing

Medium	No. explants	Number healed		
		8 ± Hrs	20 ± Hrs	Total
Saline	27	0	1	1
Glucose 100 mg%	15	12	3	15
Glucose 50 mg%	12	12	—	12
Glucose 10 mg%	15	7	7	14
Glucose 5 mg%	20	5	7	12
Fructose 100 mg%	6	0	2	2
Fructose 50 mg%	17	1	8	9
Galactose 50 mg%	12	0	6	6

this process on media containing 100 mg% and 50 mg% glucose. These media have been found by Spratt (1949a) to be adequate for the development of normal-appearing embryos. The value of the additional carbohydrate source was obvious in these explants; wounds healed completely on those media containing glucose and essentially never on those without a sugar.

On the medium containing 10 mg% (5.5×10^{-4} *M*) glucose the blastoderms began to show the effects of substrate dilution. While essentially all (14 out of 15) explants healed by 20 hours, only one half had done so at 8 hours. Compare this with those on 100 mg% and 50 mg% glucose, where practically all healing had

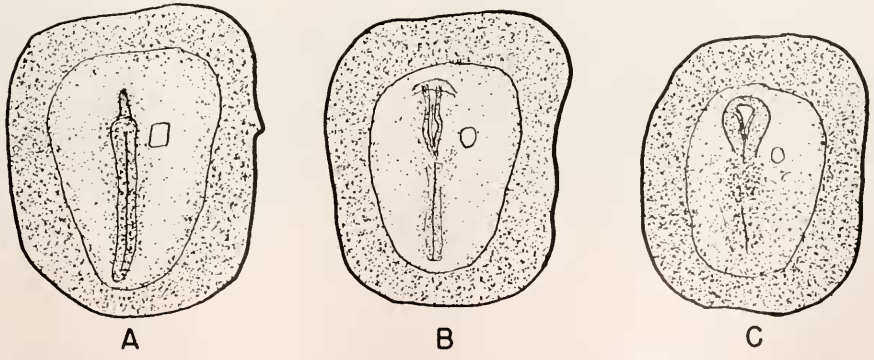

Figure 1. Camera lucida diagrams of an explant on agar-saline (control) medium to illustrate progress in wound closing and embryonic development. A = initial; B = after 8 hours; C = after 20 hours.

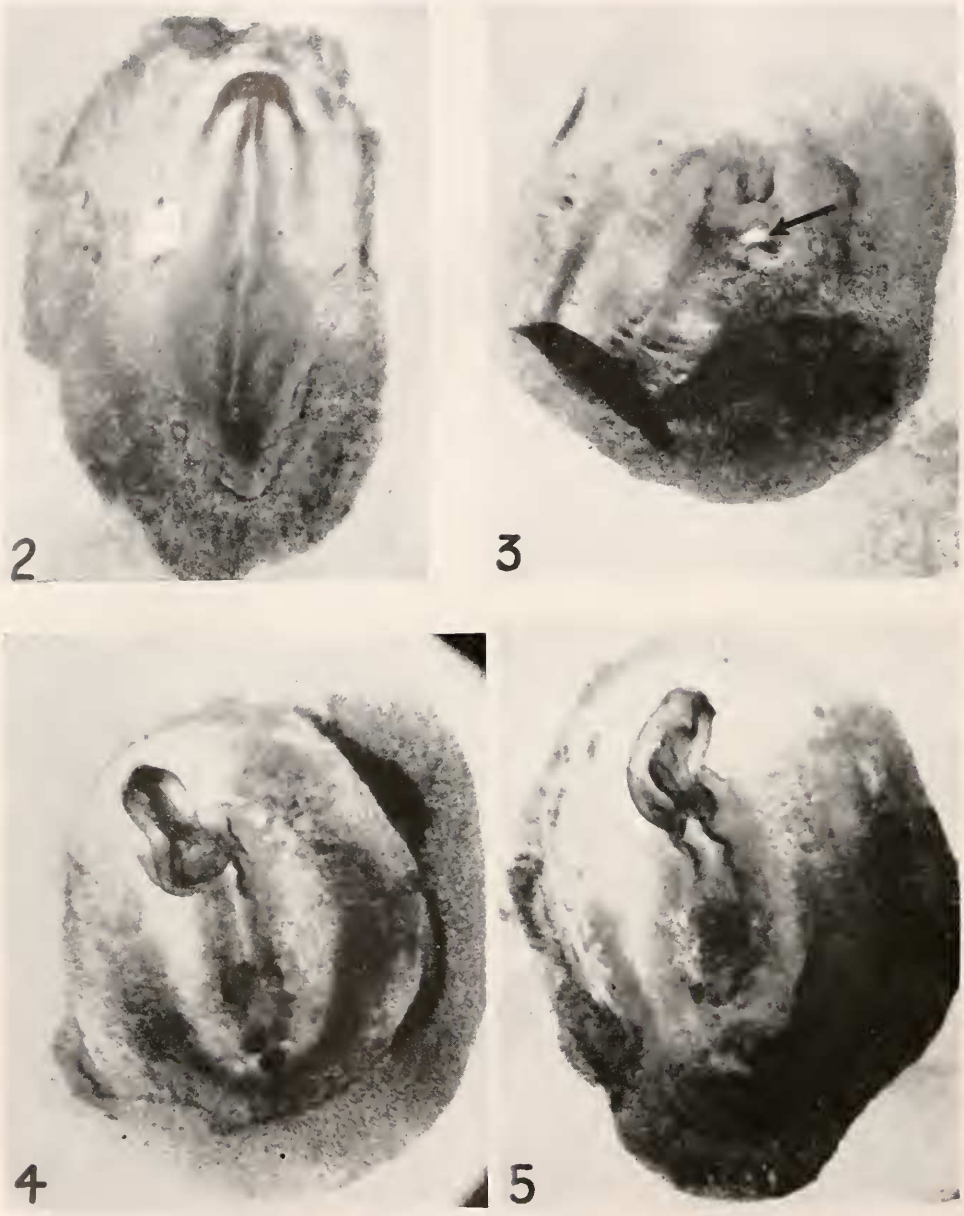

FIGURE 2. A young chick blastoderm showing the location of a wound in the pellucid area. × 20.

FIGURE 3. The appearance of a wounded chick blastoderm which had been incubated on a non-nutrient (agar-saline) medium for twenty hours. The arrow indicates an unhealed wound. × 15.

FIGURE 4. Photograph of a chick blastoderm which had been wounded and explanted on a medium containing glucose in concentration of 5 mg% for twenty hours. Note that in this

occurred by 8 hours. Of the twenty blastoderms explanted on the 5 mg% medium only twelve had healed by 20 hours, and of these only five had done so at the end of 8 hours.

The volume of the medium on which the embryos lay was very large (ca. 2 ml.) compared to the size of the blastoderms. It is safe to assume that at the end of twenty hours there was, for all practical purposes, approximately the same amount of sugar in the medium as there was initially. It is thus inconceivable that there was a significant depletion of the exogenous substrate. The argument that there may have been a localized depletion at the site of the embryo also cannot be valid, because there was a film of solution around them through which nutritional material could pass. There was thus a constant supply of substrate available to the explants. It is only logical to assume that the reason for the delay in closure or the failure to heal was that, while the total amount of sugar was ample, the concentration of it was insufficient to meet the requirements of the embryo for this purpose.

Both fructose and galactose were not as adequate as glucose as carbohydrate sources. Similarly it was found by Spratt (1949a) that they were inferior to glucose in terms of their utility in general developmental processes. This presumably reflected an inadequacy on the part of the embryos of this stage to bring these monosaccharides into the general glycolytic scheme.

Turning to the development of the embryos it was found that the controls continued to develop on the sub-minimal medium. By 8 hours the head was well undercut, neurulation was evident and somites were present. At this time the holes had closed somewhat in most cases. At 20 hours, however, there was evidence of degenerative changes. The anterior region had developed somewhat more than at 8 hours, but the node had become quite opaque, and had failed to regress (Fig. 3). When a drop of saline was placed gently on the embryos at this time, there was marked cell dispersal at the node region. This area has been generally recognized as one of high metabolic activity (Moog, 1943; Hyman, 1927), showing great sensitivity to metabolic inhibitors (Spratt, 1950b). The effects of starvation were therefore in accordance with these previous observations. While there was limited development in the embryos between 8 and 20 hours, there were no significant changes in wound sizes during this period. This point will be considered in more detail shortly. Figure 1, showing camera lucida illustrations made of an explant at time 0, 8 and 20 hours, reveals these points.

Development on glucose media in concentrations of 100 mg% and 50 mg% was fairly normal over the twenty-hour period with brain, heart and somites forming. Such media, however, were not as adequate as an albumen medium for typical embryonic development. On 10 mg% glucose the explants still continued to develop over the twenty-hour period, there being no indication of nutritional deficiency except as indicated; there was a longer period of time required for wound healing. If there was any damage to the node region, it was not readily apparent in the explants.

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embryo the wound has healed, that development is limited to the anterior end, and that the node shows signs of marked deterioration. × 15.

FIGURE 5. A chick blastoderm after twenty hours of incubation on a fructose medium (50 mg%) following injury. The same features as indicated in Figure 4 may be seen. × 15.

On the 5 mg% glucose medium there was no perceivable influence of nutritional deficiency at 8 hours, but by 20 hours these blastoderms resembled the controls (Fig. 4). While there was slight development between these time intervals, again this was limited to the head region, with lower (axial) levels showing degeneration. The somites which had formed became indistinct, the node region failed to regress and took on an opaque appearance. Illustrating details by the camera lucida became very difficult, due to the lack of translucency and the dispersal of cells. In these embryos the wounds were in all stages of closure at 8 hours. After this time about one half of the unhealed ones completed this process, as shown in Table I.

Blastoderms on fructose and galactose media showed the effects of nutritional deficiency in terms of development as well as in wound healing. These embryos behaved very similarly to those on 5 mg% glucose, again showing marked node deterioration (Fig. 5).

Pretreatment study

There was one further factor to consider in attempting to determine the minimum concentration of exogenous substrate required for wound healing. This was

TABLE II

Wound healing on minimal media after pretreatment

Medium	No. explanted	No. hrs. pretreated	No. healed after 12 ± hrs.
Glucose 10 mg%	12	5	12
Glucose 10 mg%	12	10	12
Glucose 5 mg%	18	10	12
Glucose 5 mg%	18	20	18*

* Hole filled in by loose cells during embryo degeneration.

the endogenous material present in the embryo itself. It will be noticed that even on a medium lacking any carbohydrate there was a certain amount of development. Wounds in explants on this medium also started to heal, and in one case closed completely. If, then, the effect of the carbohydrate alone was to be determined, it was necessary to minimize the endogenous substrate factor.

This was done by "pretreating" the embryos on a non-nutrient (agar-saline) medium prior to explanting them on another containing the sugar. The blastoderms were placed on the non-nutrient medium for a specified period of time, removed and wounded, and then transferred to the sugar-bearing medium. It was desirable to have the blastoderms use up most of their own available energy sources without damaging them beyond recovery.

After 8 hours of incubation there had been no apparent closure in the controls. The pretreatment period was therefore set around this interval of time. Some embryos were starved for five hours, some for ten, and some for twenty hours. Glucose concentrations of 10 mg% and 5 mg% were used, because it was around these values that the effect of deficiency became apparent.

Table II indicates the results obtained. From the data it seems that glucose in a concentration of 10 mg% was sufficient for the embryos to use in healing. This is evident even after 10 hours of pretreatment. Blastoderms explanted on a medium containing 5 mg% glucose were found, after 12 hours incubation, to have healed in approximately the same proportion as those not treated. Not included in the table were twelve embryos that were pretreated, wounded, and then transferred again to a saline-agar medium. These served as controls. None of these had healed within the twelve-hour period. From these data it may be concluded that 5 mg% glucose is near the minimal concentration that meets the requirements of the blastoderms in wound closing. Differences in ability to heal on this medium may be interpreted as an indication of variability in the embryos themselves, when explanted on synthetic media.

After twenty hours of pretreatment the node region of the explants was undergoing degeneration. When these embryos were wounded and explanted on the 5 mg% medium, it was noticed after 12 hours that the wound had apparently healed. This is indicated in Table II. When a drop of saline was gently placed over the blastoderms by means of a wide-bored pipette, however, the cells in the wound area and those at the node dispersed leaving holes in the explants. This healing was therefore not taken as normal in the sense that it was brought about by the general movements of normal tissues, but was the result of the association of cells dispersed from other areas during deterioration of the embryos.

Elsewhere (Fraser, 1953) it was mentioned that attempts to incubate the blastoderms under the medium against a cover glass failed. It was not known whether the inability of the embryos to develop was the result of insufficient oxygen or of some other agent, such as pressure of the medium on the embryo. In the course of this study the effect of anaerobiosis on wound closure was explored. Twenty embryos of 20 hours incubation were used for this purpose.

Following wounding these embryos were explanted on an agar-albumen medium in the usual manner (see Spratt, 1947). The lids of the petri dishes in which the embryos were incubated were kept elevated slightly with pieces of aluminum foil to permit the removal of oxygen. Moist cotton rings were again used in the petri dishes to maintain a moist atmosphere. The petri dishes were placed in a large desiccator into which was poured 50 ml. of 40 per cent pyrogallic acid and 100 ml. of 20 per cent KOH for the removal of oxygen. The lid was sealed immediately and the desiccator placed in the incubator.

After 12 hours the explants were removed for observation. At this time it was found that there was no embryonic development, the wounds were as large as initially made, and the blastoderms showed extensive deterioration. They had an opaque appearance masking all internal structure. When a drop of Ringer solution was gently placed on them, they tore apart with much cell dispersal, quite unlike blastoderms of a comparable age cultured on this medium under normal conditions. This indicated that normal cell-cell adhesiveness was lacking. It was apparent from this that oxygen is required for wound healing, and, in general, for all normal development. Since carbon dioxide was also removed by the alkaline pyrogallol, the effects may be in part due to its deficiency as well (Spratt, 1949b). The problem of oxygen requirement for early chick development has been considered elsewhere (Philips, 1941, 1942; Spratt, 1950a).

Discussion

There exists, as would be expected, a good correlation between the ability of the embryos to undergo development (including all of its component processes) and extra-embryonic tissue movements on various media. This correlation is not perfect, however, as evidenced by the fact that on a non-nutrient medium the blastoderms continued to develop (even though limited to the anterior region) after 8 hours of incubation, while the wounds failed to undergo any appreciable change in size beyond this time. This is a strange situation in view of the fact that one might assume that more carbohydrate (= potential energy or carbon skeleton source) would be utilized in morphogenesis, histogenesis of tissues, maintenance etc. occurring in the embryo proper than in more peripheral areas of the pellucid area, where presumably little such activity is taking place. If this assumption is correct, and we have no basis for not believing it to be so, then an answer must be sought for the observations.

It is not likely that more carbohydrate is required in tissue movements involved in wound healing, because there is more of such activity taking place in the development of the embryo itself. In this regard, however, it is interesting to note that the trunk level of the embryos failed to develop, but this is associated with, and probably a consequence of, the degeneration of the whole node region.

Since embryonic development ensues on a non-nutrient medium after general cell movements cease in the extra-embryonic region, and since it is assumed that more exogenous nutrient is required in the axial area, the suggestion is made that there may be a greater concentration of endogenous substrate localized in the embryo proper than in the outlying pellucid tissues. Although we have no direct evidence for this, such a localization is not hard to conceive in view of the general consideration that substrate and corresponding enzyme are found together. The presence of indophenol oxidase and dehydrogenase activities restricted mainly to axial tissues has been demonstrated by Moog (1943) and Spratt (1952), respectively. It is stressed, however, that the demonstration of localized enzyme activity cannot be offered as proof that there is an accumulation of its corresponding substrate.

Summary

1. Wounds produced in the pellucid area of chick embryos cultured on a non-nutrient medium failed to heal within 20 hours. Although there was little or no change in the dimensions of the holes after 8 hours, differentiation of the head region continued beyond this time.

2. On media of 100 mg%, 50 mg% and 10 mg% glucose the blastoderms healed, for the most part, within 8 hours, while development continued in the embryo proper. On a medium containing glucose in concentration of 5 mg%, however, about one half of the wounds did not heal, correlated with degenerative changes in the embryos, principally at the node, occurring between 8 and 20 hours after injury.

3. Fructose and galactose were found to be quite ineffective as carbohydrate sources for the closure of wounds and for development in general. Results using these media were comparable to those when 5 mg% glucose was utilized.

4. By pretreating the blastoderms for 5 and 10 hours on saline-agar prior to wounding, with subsequent transfer to media containing glucose, it was determined that 5 mg% glucose was approximately the minimal concentration required by the embryo for wound closure.

5. In view of the observation that embryonic development continued beyond the time when healing stopped, and because it was assumed that more carbohydrate must be required for the former to take place, it was postulated that there is a greater concentration of endogenous substrate localized in the axial tissues than in the outlying pellucid region.

LITERATURE CITED

FRASER, R. C., 1953. Studies on morphogenesis in the young chick embryo cultured *in vitro*. Unpublished thesis.

HYMAN, L. H., 1927. The metabolic gradient of vertebrate embryos. III. The chick. *Biol. Bull.*, **22**: 1–39.

MOOG, F., 1943. Cytochrome oxidase in early chick embryos. *J. Cell. Comp. Physiol.*, **22**: 223–231.

PHILIPS, F., 1941. The oxygen consumption of the early chick embryo at various stages of development. *J. Exp. Zool.*, **86**: 257–287.

PHILIPS, F., 1942. Comparison of the respiratory rates of different regions of the chick blastoderm during early stages of development. *J. Exp. Zool.*, **90**: 83–100.

SPRATT, N. T., JR., 1947. A simple method for explanting and cultivating early chick embryos *in vitro*. *Science*, **106**: 452.

SPRATT, N. T., JR., 1949a. Nutrition requirements of the early chick embryo. I. The utilization of carbohydrate substrates. *J. Exp. Zool.*, **110**: 273–298.

SPRATT, N. T., JR., 1949b. Carbon dioxide requirements of the early chick embryo. *Anat. Rec.*, **105**: 118.

SPRATT, N. T., JR., 1950a. Nutritional requirements of the early chick embryo. II. Differential nutrient requirements for morphogenesis and differentiation of the heart and brain. *J. Exp. Zool.*, **114**: 375–402.

SPRATT, N. T., JR., 1950b. Nutritional requirements of the early chick embryo. III. The metabolic basis of morphogenesis and differentiation as revealed by the use of inhibitors. *Biol. Bull.*, **99**: 120–135.

SPRATT, N. T., JR., 1952. Differentiation in reducing enzyme systems in the early chick blastoderm. *Anat. Rec.*, **113**: 602.